original reports

Cell-Free Tumor DNA Dominant Clone Allele Frequency Is Associated With Poor Outcomes in Advanced Biliary Cancers Treated With Platinum-Based Chemotherapy

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abstract

PURPOSE This investigation sought to evaluate the prognostic value of pretreatment of circulating tumor DNA (ctDNA) in metastatic biliary tract cancers (BTCs) treated with platinum-based first-line chemotherapy treatment.

MATERIALS AND METHODS We performed a retrospective analysis of 67 patients who underwent ctDNA testing before platinum-based chemotherapy for first-line treatment for metastatic BTC. For analysis, we considered the detected gene with highest variant allele frequency as the dominant clone allele frequency (DCAF). Results of ctDNA analysis were correlated with patients' demographics, progression-free survival (PFS), and overall survival (OS).

RESULTS The median age of patients was 67 (27-90) years. Fifty-four (80.6%) of 67 patients evaluated had intrahepatic cholangiocarcinoma; seven had extrahepatic cholangiocarcinoma, and six gallbladder cancers. Forty-six (68.6%) of the patients were treated with cisplatin plus gemcitabine, and 16.4% of patients received gemcitabine and other platinum (carboplatin or oxaliplatin) combinations, whereas 15% of patients were treated on a clinical trial with gemcitabine and cisplatin plus additional agents (CX4945, PEGPH20, or nab-paclitaxel). *TP53, KRAS, FGFR2, ARID1A, STK11,* and *IDH1* were the genes with highest frequency as DCAF. The median DCAF was 3% (0%-97%). DCAF > 3% was associated with worse OS (median OS: 10.8 *v* 18.8 months, $P = .032$). Stratifying DCAF in quartiles, DCAF > 10% was significantly related to worse PFS (median PFS: 3 months, $P = .014$) and worse OS (median OS: 7.0 months, $P = .001$). Each 1% increase in ctDNA was associated with a hazard ratio of 13.1 in OS when adjusting for subtypes, metastatic sites, size of largest tumor, age, sex, and CA19-9.

CONCLUSION DCAF at diagnosis of advanced BTC can stratify patients who have worse outcomes when treated with upfront platinum-based chemotherapy. Each increase in %ctDNA decreases survival probabilities.

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ASSOCIATED CONTENT

Appendix

Data Supplement

Author affiliations and support information (if applicable) appear at the end of this article.

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INTRODUCTION

Biliary tract cancers (BTCs) include intrahepatic cholangiocarcinoma (IHC), gallbladder cancer (GBC), extrahepatic cholangiocarcinoma (EHC), and ampulla of Vater cancers.[1] BTC represents 3% of gastrointestinal malignancies, with 11,980 cases expected to be diagnosed in 2021.[2,3] As BTC usually present at an advanced stage, only 20% of these tumors are considered resectable.[4] In patients with unresectable disease, the 5-year overall survival (OS) is about 4%.[5]

The survival gain with first-line chemotherapy regimens in BTC is modest since most patients develop progressive disease with a median OS of less than a year.[6] This has generated interest in using next-generation tumor genomic profiling and liquid tumor biopsy on peripheral blood to look for targetable genetic alterations.[7,8]

Circulating tumor DNA (ctDNA) has been shown to carry tumor-specific genetic or epigenetic alterations including point mutations, copy number variations, chromosomal rearrangements, and DNA methylation. This ctDNA is released into the circulation after tumor cells undergo apoptosis or necrosis.[9] Evaluation of ctDNA can identify patient-specific tumoral genetic alterations while allowing for serial monitoring of tumor genomes in a noninvasive and accurate manner.[8] Therapeutically

CONTEXT

Key Objective

In this study, we hypothesized that the dominant clone allele frequency (DCAF) on circulating tumor DNA (ctDNA) in advanced biliary tract cancer at diagnosis would be associated with overall survival (OS) and progression-free survival.

Knowledge Generated

DCAF is strongly associated with progression-free survival and OS. Each 1% increase in ctDNA was associated with a hazard ratio of 13.1 in OS when adjusting for subtypes, metastatic sites, size of largest tumor, age, sex, and CA19-9.

Relevance

DCAF at diagnosis of advanced biliary tract cancer can stratify patients who have worse outcomes when treated with upfront platinum-based chemotherapy. Each increase in %ctDNA decreases survival probability.

relevant alterations were seen in ctDNA in 55% of patients with BTC.[8] Because of these findings, the strategy is being used in the setting of advanced disease for treatment selection.[10] Furthermore, it has also been used as an early marker of response to treatment and to track mechanisms of acquired resistance.[11]

In colon and breast cancer, ctDNA has been used to predict response to treatment and prognosis in the adjuvant and neoadjuvant setting, respectively.[12,13] One marker of interest is variant allele frequency (VAF), which is the number of mutant molecules over the total number of wild-type molecules at a specific location on the genome. Pairawan et al[14] showed that VAF is a surrogate marker of tumor burden and maximum VAF (VAFmax) correlated negatively with prognosis and survival in metastatic cancer.

In this study, we hypothesized that the dominant clone allele frequency (DCAF) on ctDNA in BTC would be associated with OS and progression-free survival (PFS) and can serve as a surrogate of disease volume and severity. In addition, we looked at the relationship of DCAF to treatment response with first-line platinum-based chemotherapy and clinical demographics.

MATERIALS AND METHODS

Patients

From July 2016 through June 2020, 67 patients with advanced BTC underwent ctDNA testing at diagnosis using an available assay (Guardant Health, Inc, Redwood City, CA). All the patients received care at the Mayo Clinic Cancer Center in Arizona and Florida. The analysis from this cohort was reviewed and approved by the Mayo Clinic institutional review board. Clinical and demographic information of all patients is included in Table 1.

Comprehensive Genomic Testing in Plasma

Circulating tumor DNA was extracted from whole blood. ctDNA fragments, both leukocyte-derived and tumor-derived, were simultaneously sequenced. The VAF was calculated as the proportion of ctDNA harboring the variant in a background of wild-type ctDNA. Analytical information, bioinformatics analysis, and Guardant360 database have been previously described.[15,16]

Outcomes

Assessments regarding response to therapy (complete response [CR], partial response [PR], stable disease, and disease progression) were retrospectively collected by review of patient's charts. Positive response to therapy was considered PR and CR by RECIST. Disease control rate was determined on the basis of CR, PR, and stable disease. PFS was determined during treatment with chemotherapy and after without disease progression. OS was determined by the time of diagnosis of advanced disease until death or last day of follow-up for patients on treatment and alive.

Statistical Analysis

We summarized categorical data as frequency counts and percentages, and continuous measures as means, standard deviations, medians, and ranges. Categorical variables were compared using the chi-square test or Fisher's exact test. Continuous variables were compared using the one-way analysis of variance test or Kruskal-Wallis test. Multivariate logistic regressions were performed to assess the association of ctDNA with response rate and disease control rate with adjustment for disease subtype, age, sex, CA19-9, lesion size, and metastatic site. The distributions of time-to-event outcomes were estimated using the Kaplan-Meier methods and compared between low and high ctDNA dichotomized by the median DCAF (ie, low $< 3\%$ *v* high $\geq 3\%$ ctDNA) using the log-rank test. Hazard ratios (HRs) and 95% CIs were estimated using a multivariate Cox model adjusting for disease subtype, age, sex, CA19-9, lesion size, and metastatic site. Sensitivity analysis was performed to explore either 3 quantiles ($\leq 33\%$ percentile, 34%-66% percentile, and $> 66\%$ percentile) or quartiles as the cutoffs in DCAF.

Ethics

The study was reviewed and approved by the Mayo Clinic Institutional Review Board. The informed consent was waived after IRB evaluation. This study was conducted in accordance with the Declaration of Helsinki.

TABLE 1. Demographic Characteristics

	Overall (N = 67)
Age at diagnosis, years	
Median (range)	67 (27-90)
Sex, No. (%)	
Male	25 (37.4)
Female	42 (62.6)
Diagnosis, No. (%)	
EHC	7 (10.4)
Gallbladder carcinoma	6 (9.0)
IHC	54 (80.6)
Grouping by genomic alterations, No. (%)	
FGFR2	9 (13.4)
ERBB2	6 (9.0)
IDH1	11 (16.4)
KRAS	9 (13.4)
Not detectable	1 (1.5)
Others (*TP53, CDKN2A, BRAF, STK11, APC, RET, ARID1A, EGFR*, and *ATM*)	31 (46.3)
Treatment, No. (%)	
Gemcitabine plus cisplatin	46 (68.6)
Gemcitabine plus cisplatin plus CX-4945	6 (8.95)
Gemcitabine plus oxaliplatin	6 (8.95)
Gemcitabine plus carboplatin	5 (7.5)
Gemcitabine plus cisplatin plus nab-paclitaxel	3 (4.5)
Gemcitabine plus cisplatin plus PEGPH20	1 (1.5)
Size of largest lesion, cm	
Median (range)	6 (2-19)
Metastatic sites, No. (%)	
Liver	24 (35.8)
Extrahepatic only (eg, lung, bones, peritoneum, and lymph nodes)	4 (6)
Liver + extrahepatic	39 (58.2)
Pretreatment CA19-9 level, U/mL	
Median (range)	103 (1-103,198)
Pretreatment maximum mutant allele frequency, %	
Mean CI	10 (6 to 14)
Median (range)	3 (0-94)
Q1, Q3	1, 10
Tumor response (PR + CR), No. (%)	
No	34 (57.6)
Yes	25 (42.4)
Disease control (PR + CR + SD), No. (%)	
No	21 (35.6)
Yes	38 (64.4)
PFS events, No. (%)	
No	31 (46.3)
Yes	36 (53.7)
OS events, No. (%)	
No	28 (41.8)
Yes	39 (58.2)

(Continued in next column)

TABLE 1. Demographic Characteristics (Continued)

Abbreviations: CA19-9, cancer antigen 19-9; CR, complete response; EHC, extrahepatic cholangiocarcinoma; IHC, intrahepatic cholangiocarcinoma; OS, overall survival; PFS, progression-free survival; PR, partial response; Q1, quantile 1; Q3, quantile 3; SD, stable disease.

RESULTS

Patient Demographics

A total of 67 patients were included in the analysis. 80.6% (54) had IHC, 10.4% (seven) of patients had EHC, and 9% (six) had GBC. All patients included had ctDNA collected before the first-line chemotherapy regimen for advanced disease. The median age of all patients was 67 (27-90) years, and the majority were female (62.6%). All patients were treated with platinum-based chemotherapy regimens as first-line treatment. Most patients (68.6%) were treated with cisplatin plus gemcitabine, and 11 (16.4%) patients received gemcitabine plus other platinum (carboplatin or oxaliplatin) combinations, whereas 10 (15%) patients were treated on a clinical trial with gemcitabine and cisplatin plus additional agents (CX4945, PEGPH20, or nab-paclitaxel). The median size of largest lesion was 6 cm (2-19 cm), and more than half (58.2%) had multiple metastatic sites including liver and extrahepatic sites. Lungs, bones, lymph nodes, and peritoneum were the sites with most extrahepatic metastasis identified. Other clinical information is given in Table 1.

Several potential targetable genes were detected with ctDNA including *FGFR2, HER2, IDH, MET, EGFR, BRAF*, and *KRAS*. A higher prevalence of *TP53* was observed among the three subtypes. Homologous recombinant repair genes were identified in IHC and EHC, including *ATM* and *BRCA2*. Prevalence of all genomic alterations according to primary tumor is presented in Figure 1.

TP53, KRAS, FGFR2, ARID1A, STK11, and *IDH1* were the genes with highest VAF as dominant clone (Fig 2). Most *ERBB2 (HER2)* genomic alterations detected were amplifications, identified in four patients. Other genes with detected amplifications included *KRAS, EGFR, BRAF, MET, CCNE1, CCND1, CCND2, MYC, FGFR1, FGFR2, CDK4, CDK6, PIK3CA,* and *AR*. For analysis, we considered the detected genomic alteration with the highest VAF as the DCAF.

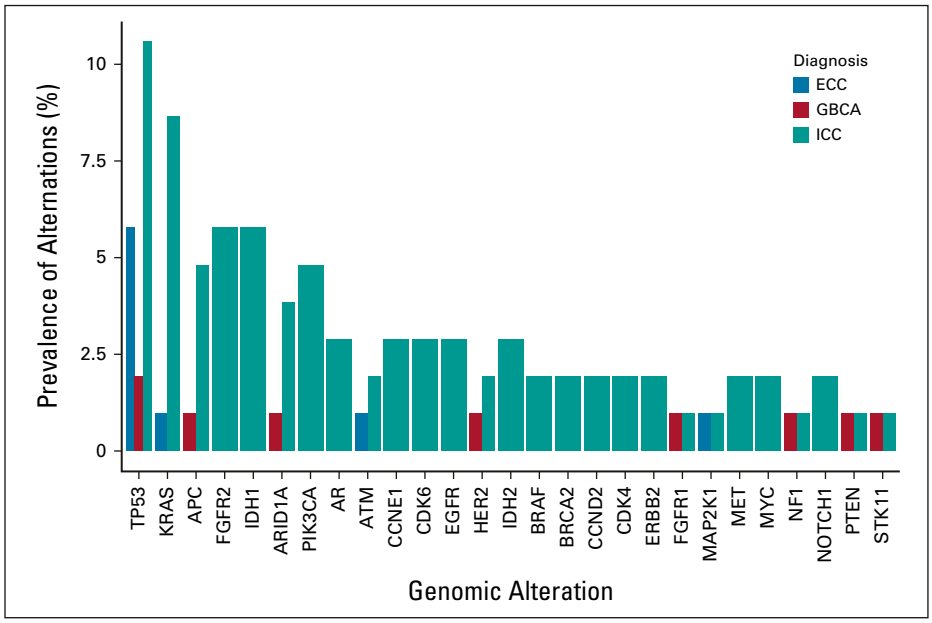

FIG 1. Prevalence of genomic alterations according to primary tumor. ECC, extrahepatic cholangiocarcinoma; GBCA, gallbladder cancer; ICC, intrahepatic cholangiocarcinoma.

DCAF and Prognostic Factors

One patient with no tumor genomic alteration detected was excluded from this analysis. The median DCAF was 3% (0%-97%). DCAF > 3% was associated with inferior PFS (median PFS: 4.7 *v* 7.7 months, *P* = .087; Data Supplement) and significantly worse OS (median OS: 10.8 *v* 18.8 months, *P* = .032. Fig 3).

We further analyzed DCAF using either three quantiles or quartiles as the cutoffs. DCAF distributed across three quantiles (Q1: ctDNA ≤ 1%, Q2: ctDNA 1%-7%, and Q3: ctDNA ≥ 7%) was significantly associated with PFS (*P* = .022), with a shorter median PFS of 3.2 months for patients with ctDNA ≥ 7%, compared with 10.5 months for patients with ctDNA ≤ 1% and 10.7 months for patients with ctDNA 1%-7% (Data Supplement). DCAF distributed across three quantiles was not statistically associated with OS differences (*P* = .065; Data Supplement). DCAF divided by quartiles (Q1: ctDNA ≤ 0.6%, ctDNA Q2: 0.6%-3%, ctDNA Q3: 3%-10%, and ctDNA Q4: ≥ 10%) was significantly associated with both PFS (*P* = .014; Fig 4) and OS (*P* = .001; Fig 5).

Each 1% increase in ctDNA is associated with a hazard ratio of 13.1 in OS when adjusting for primary tumor, size of the largest lesion, metastatic sites, sex, age, and CA19-9 (Appendix Table A1). No significant differences in response or disease control rate to chemotherapy were observed in

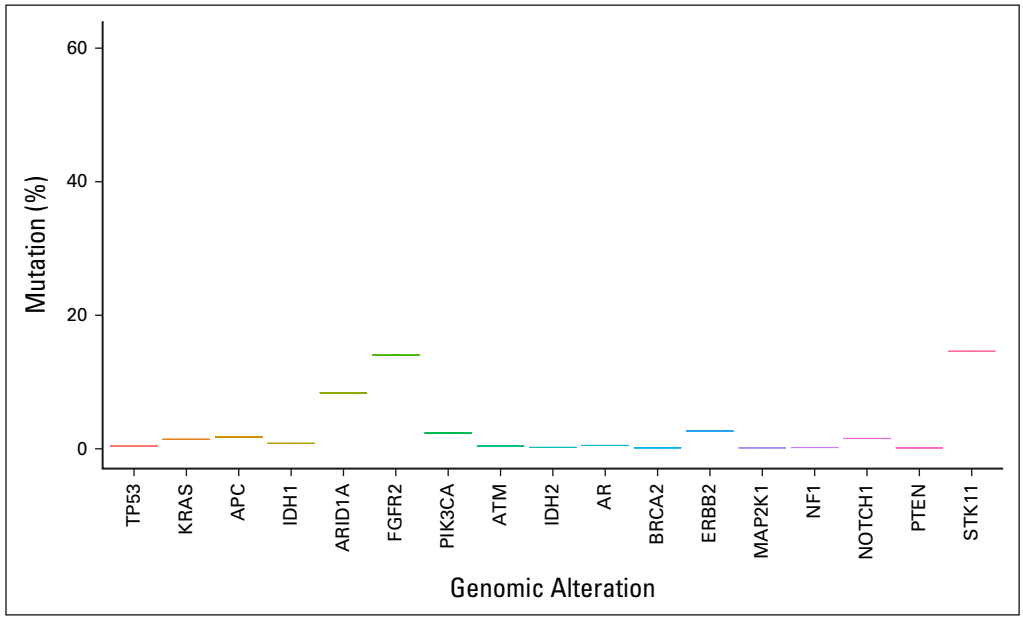

FIG 2. Variant allele frequency of detected genes.

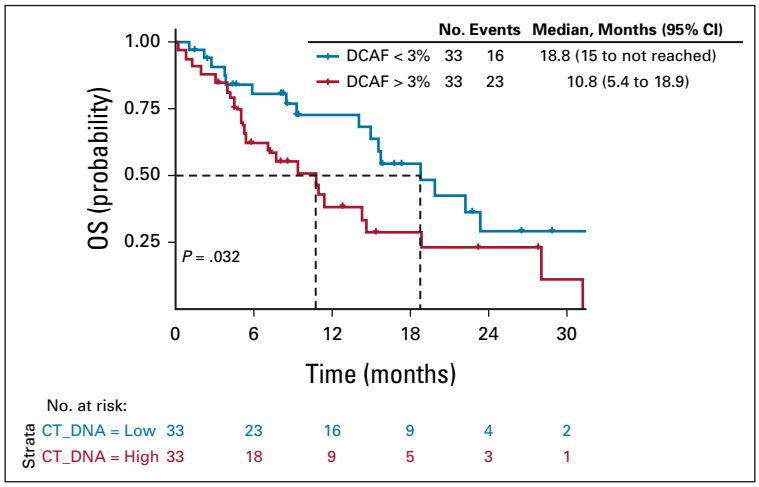

FIG 3. Kaplan-Meier curve for OS by DCAF > 3%. DCAF, dominant clone allele frequency; OS, overall survival.

patients with low or high ctDNA (Data Supplement). No statistical significance was found between DCAF and the presence of potential actionable targets including *FGFR2, IDH1/2, ERBB2,* and *KRAS* (Data Supplement). No association between ctDNA and survival in the context of *FGFR2* alterations or *IDH* mutation was identified (Data Supplement).

The interaction between CA19-9 and DCAF was not statistically significant (OS: $P_{interaction} = 0.12$; PFS: $P_{interaction} = .06$). Although cholangiocarcinoma patients with high DCAF and high CA19-9 (DFCA ≥ 3%, CA19-9 ≥ 100) had worst OS, no statistical significance was found for PFS ($P = .19$) or OS ($P = .13$; Data Supplement).

DISCUSSION

In this study, we assessed whether the highest VAF detected by ctDNA, namely, DCAF, could be a prognostic factor in patients with advanced BTC at diagnosis. On the basis of the findings, patients with DCAF > 3% at diagnosis had worse OS when treated with upfront platinum-based chemotherapy. Furthermore, DCAF> 10% was significantly related to worse PFS and OS. However, no differences in response rate were observed among patients with high or low DCAF. Moreover, ctDNA proved to be an independent factor related to OS in multivariate analysis. Collectively, these data suggest a prognostic and not predictive role for DCAF in patients with advanced BTC undergoing platinum-based therapy.

The landscape of ctDNA genomic alterations of BTCs has already been previously described.[8,17] Similar to our findings, these studies included more patients with IHC and the genes with the highest detection with ctDNA included principally *KRAS, TP53, FGFR2, IDH1,* and *ARID1A.*[8,17] In our cohort, we observed different patterns of prevalence, with *ATM* and *MAP2K1* detected in EHC and *ERBB2* and *NF1* and *PTEN* in GBC.

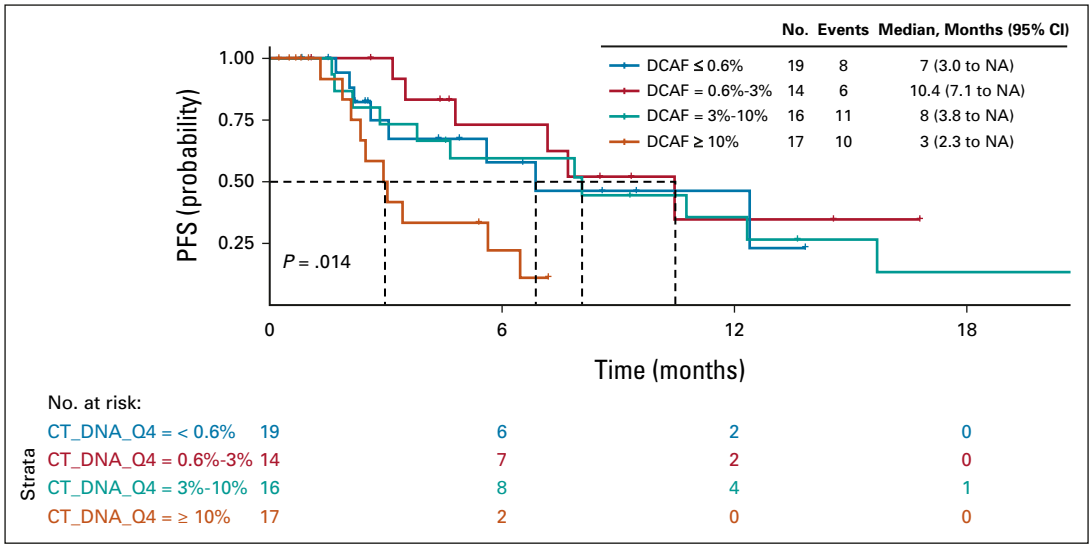

FIG 4. Kaplan-Meier curve for PFS by ctDNA. ctDNA, circulating tumor DNA; DCAF, dominant clone allele frequency; PFS, progression-free survival.

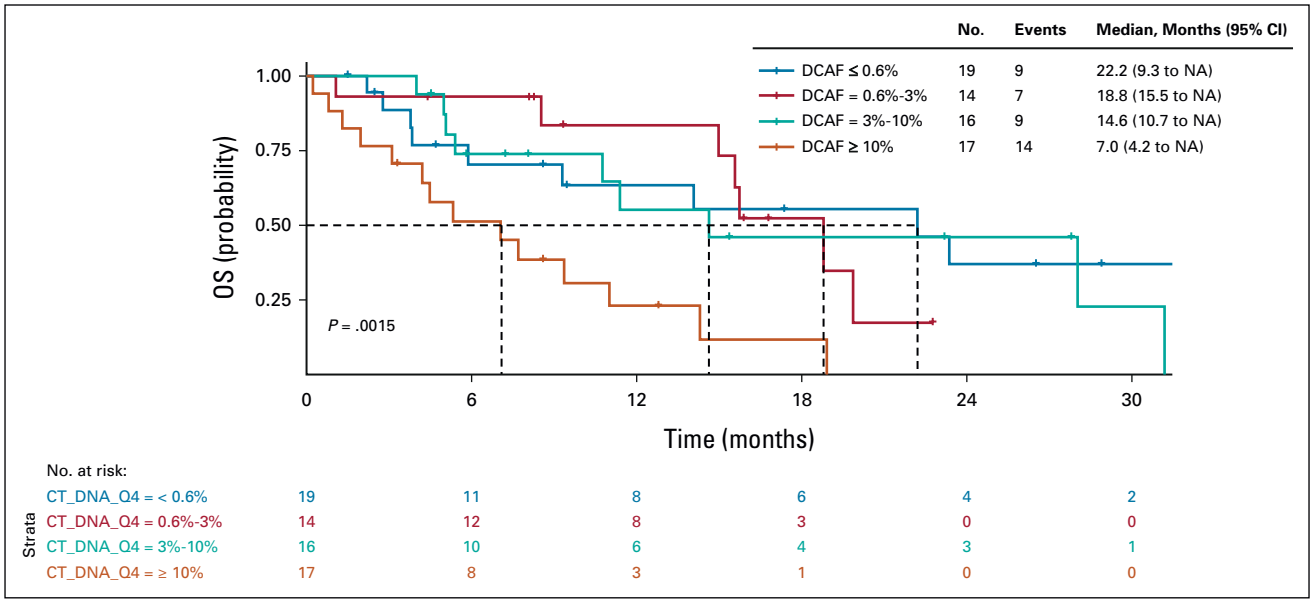

FIG 5. Kaplan-Meier curve for OS by ctDNA. ctDNA, circulating tumor DNA; DCAF, dominant clone allele frequency; OS, overall survival.

VAF is related to outcomes, which is more prominent in metastatic disease and is associated with tumor volume.[14,16,18] In metastatic pancreatic cancer, detectable ctDNA and high VAF were associated with worse OS.[18-20] Prognostic significance was observed in other solid tumors including colorectal cancer, breast cancer, and prostate cancer.[21-23] Little is known about VAF and prognosis in BTCs. Lower values of VAF were associated with prolonged PFS in a cohort of 24 patients with cholangiocarcinoma.[18] Considering the highest VAF value, we showed that the DCAF> 3% is related to numerically inferior PFS (but not statistically significant) and worse OS in patients with BTCs treated with standard upfront platinum-based chemotherapy for advanced disease. Interestingly, the DCAF was determined by multiple different genes among the cases, including *TP53, KRAS, FGFR2, ARID1A, STK11,* and *IDH1*, suggesting as previously stated by other colleagues that the highest VAF would be a surrogate of disease burden not related specifically to the gene detected. In agreement with this, evaluating the presence of specific genes of interest in the overall analysis of ctDNA, we did not find any association with DCAF and possible targetable genes including *FGFR2, ERBB2, IDH1,* and *KRAS.*

Prognostic factors related to PFS and OS in advanced BTCs were evaluated from the ABC-02 trial and an international data set.[24] In this analysis, the authors evaluated prognostic factors in a combined sample size of more than 1,000 patients.[24] Although the results suggest multiple factors in multivariate analysis including hemoglobin, sex, and neutrophils, receiver operator curve analysis suggested that the model generated had a limited prognostic value.[24] Even the primary tumor site was not significant, in contrast to the findings of other groups.[25] After multiple efforts evaluating scores and factors to prognostication of advanced BTCs,[26-28] the ability to predict prognosis needs improvement. In our analysis, the OS impact of ctDNA was observed after stratifying with other possible prognostic factors including size of largest lesion, locally advanced/metastatic designation, primary tumor location, metastatic sites, sex, cancer antigen 19-9, and age. Evaluating ctDNA as a continuous variable, higher values are related to inferior survival probabilities. On the basis of the findings, ctDNA and DCAF could be a reliable assay to collect as a prognostic instrument in prospective trials.

Considering the investigative nature of ctDNA in BTCs, larger, multicentered prospective studies would be necessary to address the application of ctDNA in various disease assessment junctures, considering early diagnosis, minimal residual disease assessment, monitoring in advanced stages during systemic treatment, and assessment of mutations that are associated with resistance during treatment with targeted therapies. Future application of ctDNA and DCAF in metastatic disease would be a tool for genomic profiling in prospective trials, can be a surrogate of disease volume, and will assist in stratification of patients with advanced BTC in randomized studies, beyond currently known factors such as locally advanced vs. metastatic disease and CA19-9 levels. DCAF could also be used for selecting patients eligible for more intensive regimens of chemotherapy on the basis of higher levels of ctDNA.

Some limitations of this study include the number of patients, limited institution aspect, inherent limitations associated with a targeted gene panel, and the retrospective nature of data collection. Furthermore, most of the patients included had tumor arising from the

intrahepatic duct. This limitation is shared with studies in BTCs, and efforts for future studies should be made to include patients with extrahepatic and gallbladder carcinomas in initiatives for genomic profiling and ctDNA. Despite the limited number of patients, strong association of ctDNA and OS was observed and provides the impetus for broader evaluation. This study only evaluated patients treated with upfront chemotherapy. Although this is the standard of care in the first-line setting, at this time, multiple trials are evaluating targeted treatments including FGFR2 inhibitors in the first-line therapy for advanced disease and prospective studies may need to account for the emerging therapeutic landscape in BTCs. In this study, ctDNA collection was evaluated before first-line platinum-based chemotherapy and was not powered to evaluate DCAF for further lines of systemic treatment. On the other hand, as stated previously, the presence of targetable genes had no association with DCAF results and impact of ctDNA on OS.

In conclusion, ctDNA is a powerful prognostic tool in advanced BTCs. DCAF at diagnosis of advanced disease for patients who would receive platinum-based systemic therapy identifies patients with a worse prognosis. ctDNA should be evaluated in prospective trials as a stratification factor for advanced disease and as a surrogate for tumor burden.

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DISCLAIMER

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH.

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K.M. and M.J.B. contributed equally to this work.

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DATA SHARING STATEMENT

The data sets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Manuscript writing: All authors
Final approval of manuscript: All authors
Accountable for all aspects of the work: All authors

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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APPENDIX

TABLE A1. Multivariate Analysis for Overall Survival

Prognostic Factors	HR	95% CI	*P*
Location of primary: non-IHC *v* IHC	1.54	0.46 to 5.21	.484825
Age at diagnosis, 10 years	1.18	0.83 to 1.67	.359703
Pretreatment CA19-9 level (≥ 100 U/mL)	2.08	0.96 to 4.53	.063824
ctDNA (%)	13.07	1.2 to 142.32	.034866
Sex	1.24	0.57 to 2.72	.588142
Lesion size	1.02	0.92 to 1.13	.762273
Metastatic site: extrahepatic (ref: liver)	0.66	0.08 to 5.47	.700660
Metastatic site: liver + extrahepatic (ref: liver)	1.09	0.49 to 2.43	.831125

Abbreviations: CA19-9, cancer antigen 19-9; ctDNA, circulating tumor DNA; IHC, intrahepatic cholangiocarcinoma; HR, hazard ratio; ref, reference.

